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Controlled release of quercetin from HPMC/gellan gum hydrogel for inhibiting melanogenesis in murine melanoma cells

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Abstract−Although quercetin inhibits melanogenesis, it is cytotoxic at high concentrations. Recent studies have actively investigated carriers for the controlled release of functional drugs at desired concentrations. In this study, porous hydrogel carriers were prepared using hydroxypropyl methylcellulose (HPMC) and gellan gum for the controlled release of quercetin, which acts as a whitening agent. The physical properties and release characteristics of quercetin from HPMC and gellan gum were measured. In a toxicity test using B16F10 melanoma cells, quercetin decreased cell viability at concentrations above 20 μM. Therefore, we used the HPMC (H)/gellan gum (G) hydrogel with H1/G9, H2/G8, H3/G7, H4/G6, and H5/G5 (w/w) ratios at a final solid content of 4% to control quercetin release at a non-cytotoxic level. Stiffness of the hydrogel increased, and the pore size became finer with a higher gellan gum content. Quercetin release rate from the HPMC/gellan gum hydrogel decreased with increasing content of gellan gum; for example, the release rate of quercetin from the H1/G9 hydrogel was approximately half of that from the H5/G5 hydrogel. These results suggest that the HPMC/gellan hydrogel may be a useful delivery vehicle to release quercetin, a whitening agent, at non-cytotoxic concentrations.

Keywords: Anti-melanogenesis, Quercetin, Drug Release, HPMC, Gellan Gum, Hydrogel

INTRODUCTION

Melanin is a macromolecule with reddish-brown to dark-brown color that is synthesized in the endoplasmic reticulum of melanocytes [1]. Melanin protects cells from ultraviolet light, preventing skin cancer and aging [2]. However, excessive melanin accumulation causes skin conditions such as spots and freckles [3]. Therefore, interest in whitening cosmetics that inhibit melanin production is steadily increasing. Kojic acid and azelaic acid are frequently used in whitening cosmetics; however, side effects, such as skin irritation have been reported [4]. In recent years, safe whitening substances that have a strong whitening effect but no side effects have been explored in natural products. Flavonoids, a type of polyphenol, have a whitening effect [5]. Quercetin is a well-known flavonoid (Fig. 1(a)) that is distributed in many plants and exerts pharmacological actions, such as antioxidant and anticancer effects [6]. Quercetin inhibits the enzymatic oxidation of L-DOPA by chelating the copper atoms of the enzyme and inhibits the activity of tyrosinase, the most important enzyme involved in melanin synthesis [5]. Although many flavanols function as copper chelators and tyrosinase inhibitors because of their structural features, quercetin is particularly effective at inhibiting tyrosinase [7]. However, the use of quercetin in whitening cosmetics is limited because of its cytotoxicity at high concentrations [8]. Development of non-toxic formulations of quercetin for cosmetic application requires more research.

Hydroxypropyl methylcellulose (HPMC) is a thermo-responsive polymer that dissolves in water at low temperatures but changes its state at high temperatures, resulting in the breakdown of hydrogen bonds and methoxy groups. Therefore, HPMC at high temperatures is characterized by the formation of a three-dimensional hydrogel network owing to the increase in hydrophobic interactions and sol-gel transition depending on temperature [9]. Based on these properties, HPMC can be used as a carrier in drug-release systems; drug diffusion from HPMC is highly dependent on the matrix swelling ratio and dissolution [10]. HPMC hydrogels formed at high temperatures show diminished hydrophobic interaction at 37 °C and weakened gel strength. During this process, the HPMC hydrogel expands and dissolves, simultaneously resulting in drug release [11]. Anionic polysaccharides can reduce the gelation time of the HPMC hydrogel through the salting-out effect and enable gelation at lower temperatures. This principle allows gellan gum, an anionic polysaccharide, to control matrix expansion and dissolution, as well as drug release from the HPMC hydrogel [12]. Further, gellan gum is an ion-responsive polymer that gels through cross-linking with cations, and its pore size can be controlled by the degree of cross-linking (Fig. 1(b)) [13].

Here, we produced an HPMC/gellan gum hydrogel matrix to effectively sequester and release quercetin at a sub-toxic concentration. At high temperature, a quercetin-captured hydrogel was prepared, which was swelled based on the characteristics of HPMC at room temperature to induce drug release. Further, the addition of gellan gum increased the hydrogel stiffness and reduced elation time to control the quercetin release rate. HPMC (H)/gellan gum (G) hydrogel with 4% final solid content was prepared at H1/G9, H2/

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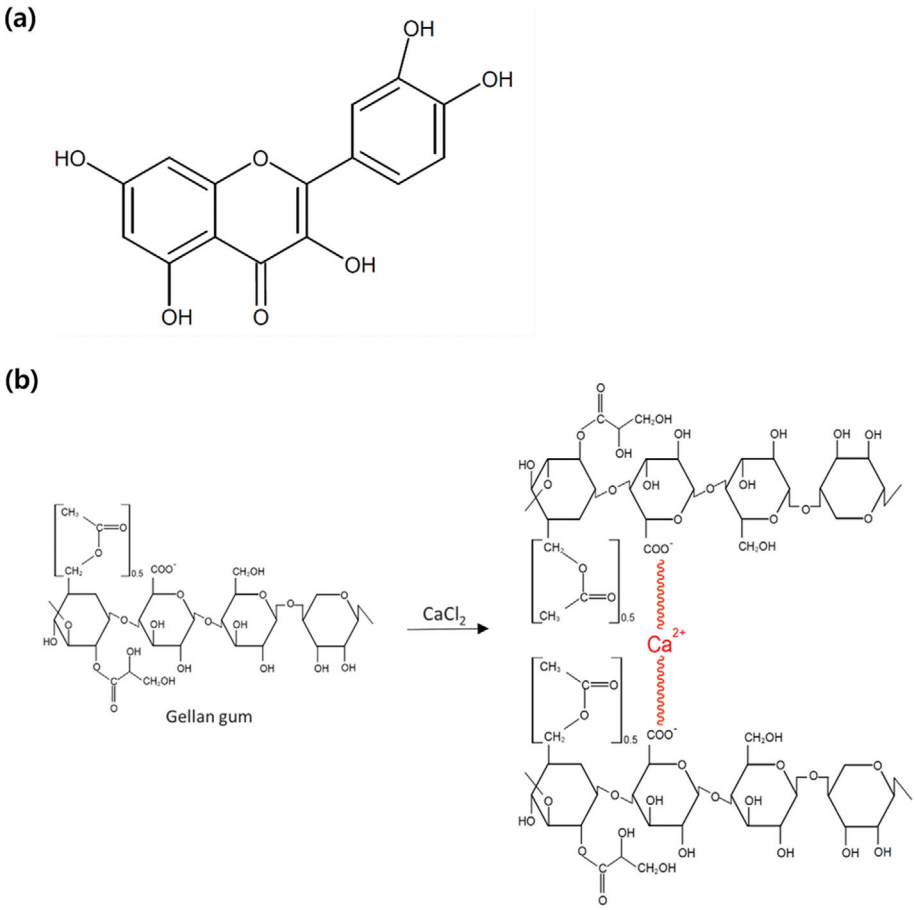

Fig. 1. (a) Structural formula of quercetin (b) Crosslinking of gellan gum with calcium ions.

G8, H3/G7, H4/G6, and H5/G5 ratios, and their physical properties and controlled drug release from the hydrogels were evaluated.

MATERIALS AND METHODS

1. Preparation of the Hydrogel

HPMC powder (AN6, LOTTE Fine Chemical) and gellan gum powder (high acyl, Kelcogel) were mixed at ratios of 5/5, 4/6, 3/7, 2/8, and 1/9 (w/w). The final concentration of the HPMC/gellan gum solution in distilled water was 4% (w/v). The gelation reaction is a two-step process. First, the HPMC/gellan gum solution was agitated at 100 °C and 650 rpm for 15 min to increase the hydrophobic interactions of HPMC. When we added quercetin in the second step, 6 mM calcium chloride ($CaCl_2$) solution was added to induce cross-linking between calcium chloride and gellan gum.

2. Physical Properties of the Hydrogel

Gelation time was measured using the vial tiling method when the gel solution did not flow for one minute.

3. Scanning Electron Microscopy

All samples were gelled and lyophilized for 48 h under vacuum to maintain their porous structure. The lyophilized sample was immediately frozen in liquid nitrogen and the surface was coated with silver for 120 s. Finally, scanning electron microscopy (SEM) images were acquired using a Hitachi S-4300 instrument to observe the hydrogel surface.

4. Stiffness

All samples were prepared with a size of 10 cm^2×3 mm, and stiffness was measured using a texture analyzer (CT3-4500, LOTTE Fine Chemical).

5. Cell Culture

Murine B16F10 melanoma cells were purchased from the Korean Cell Line Bank and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, USA) and 1% penicillin streptomycin (Gibco) at 37 °C in 5% CO_2. Cells were cultured in collagen-coated T-75 flasks, and experiments were performed using cells at passage numbers 13-15.

6. Cytotoxicity of Quercetin

The cytotoxicity of quercetin (Bamboo extraction, CHOSUN UNIVERSITY) was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (MTT, Sigma-Aldrich, USA). B16F10 melanoma cells were seeded at 10,000 cells/well in a 96-well plate (30096, SPL Life Science) for 24 h and treated with different concentrations of quercetin (0, 5, 10, 20, 30, 50, and 100 μM) for 24 h. Then, 2.5 mg/mL MTT solution was added at 100 μL per well and incubated at 37 °C for 4 h. The supernatant was removed and 200 μL of DMSO was added to each well. Finally, absorbance was measured using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA) at 540 nm.

7. Melanogenesis Inhibition by Quercetin

B16F10 melanoma cells were seeded at a density of 40,000 cells/well in a 6-well plate (30006, SPL Life Science) for 24 h and treated with 1 μM α-MSH and different concentrations of quercetin (0, 10, 20, 50, 80, and 100 μM). After 24 h, the cells were washed and harvested in PBS and centrifuged at 10,000 rpm for 5 min; then, 1 N NaOH was added to dissolve the pellet at 60 °C for 2 h. The absorbance of the dissolved pellets was measured using a microplate reader at 405 nm. Melanin content was compared with that of the quercetin-free group, and arbutin was used as a positive control.

8. Cytotoxicity of Hydrogel

To obtain DMEM containing hydrogel extract, 2 mm thick hydrogel was prepared in six wells and 3 mL of DMEM was added and stored at 37 °C for 24 h. B16F10 melanoma cells were seeded into a 96-well plate at 10,000 cells/well. After stabilization for 24 h, medium containing the hydrogel extract was added. Cells were incubated with the hydrogel extract for 24 h and 72 h with fresh medium replacement each day. Cells exposed to hydrogel extracts were analyzed using the EZ-Cytox Cell Viability Assay Kit (Daeil Labservice, Korea) for 1 h at 37 °C and absorbance was measured at 450 nm using a microplate reader.

9. Water Uptake in Hydrogel

HPMC/gellan gum hydrogel samples were prepared in a 35 mm Petri dish to a thickness of 2 mm, and the initial weight was measured. Then, 2 mL of PBS was added to a Petri dish containing hydrogels and swelled at 37 °C. The PBS was removed at regular intervals, the sample was weighed, and replaced with fresh PBS. The water uptake of the HPMC/gellan gum hydrogels was calculated using the following equation, where H_s is the weight of the swollen hydrogel (g) and H_d is the weight of the dried hydrogel (g).

$$\text{Water uptake of H/G hydrogel (\%)} = \frac{H_s - H_d}{H_d} \times 100$$

10. Release of Quercetin

The HPMC/gellan gum hydrogel encapsulating 100 μM quercetin was prepared in a 35 mm Petri dish to a thickness of 2 mm. Next, 2 mL of PBS solution was added and stored at 37 °C. The PBS solution was harvested each time and replaced with fresh PBS. The absorbance of the harvested PBS solution was measured at 369 nm, the detection wavelength for quercetin. The released quercetin content was quantified using a standard curve. Drug release is the most frequent during the first 2 h of release; therefore, quercetin release was measured every 30 min for 2 h.

11. Statistical Analysis

All experimental measurements were independently repeated three or more times, and the results expressed as mean±SD. The significance of the difference between the test and control groups was evaluated using Student's *t*-test (two-tailed).

RESULTS

1. Physical Properties of Hydrogel

To investigate the effect of HPMC/gellan gum ratio on the gelation of the hydrogels, gelation time was measured according to the ratio of HPMC and gellan gum (Fig. 2). The H5/G5 hydrogel with the highest HPMC ratio and the lowest gellan gum ratio required

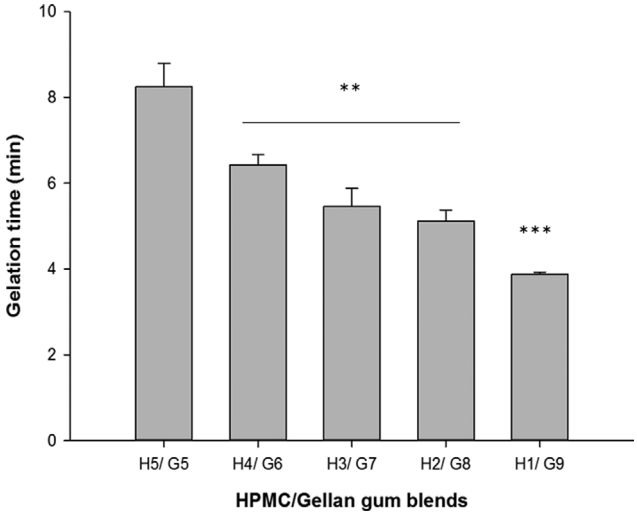

Fig. 2. Effect of the HPMC and gellan gum concentration ratio on gelation time. The total solid concentration of each hydrogel was 4% (w/v), and $CaCl_2$ was added at the same concentration of 6 mM. The results were compared using the H5/G5 hydrogel with the lowest concentration of gellan gum as the control (n=3, mean±SD, *: p<0.05, **: p<0.01 and ***: p< 0.005).

8.249±0.545 min to fully gel. Conversely, the gelation time of the H1/G9 hydrogel with the lowest ratio of HPMC and highest ratio of gellan gum was 3.872±0.054 min, and the gelation time was approximately twice as fast as that of the H5/G5 hydrogel. Overall, gelation time was shortened as HPMC content decreased, and gellan gum content gradually increased. HPMC/gellan gum hydrogels prepared at different concentration ratios were lyophilized and coated, and the surface was observed using SEM (Fig. 3). The HPMC/gellan gum hydrogel samples showed a smaller pore size and uniform structure with a higher concentration of gellan gum. The change in hydrogel stiffness with changes in the HPMC and gellan gum ratio was also measured (Fig. 4). Among all hydrogels measured, the lowest stiffness was 5.6667±0.424 g at the ratio of H5/G5, with the lowest ratio of gellan gum addition. Conversely, the hydrogel with the highest stiffness was 21.6667±3.313 g at the ratio of H1/G9 with the highest gellan gum content. Overall, the hydrogel stiffness increased significantly with the addition of gellan gum.

2. Effect of Hydrogel Extract on Cell Viability

Fig. 5 shows the effect of the HPMC/gellan gum hydrogel extract on cell viability. Cells exposed to the gel extract for 24 h showed a viability of 100% or more under all conditions. Cell viability was slightly reduced compared to that of the control, but remained at 95% or more under all conditions.

3. Cell Viability and Anti-melanogenic Effect of Quercetin

To measure the cytotoxicity of quercetin, melanoma cells were treated with quercetin (5-100 mM), and cell viability was measured after 24 h (Fig. 6(a)). In this study, cytotoxicity was not observed when the quercetin concentration was below 20 μM. However, when this quercetin concentration was exceeded, cytotoxicity was observed. After treatment with 30, 50 m, and 100 μM was treated, cell viability was reduced by 19.46, 35.19, and 43.55%, respectively.

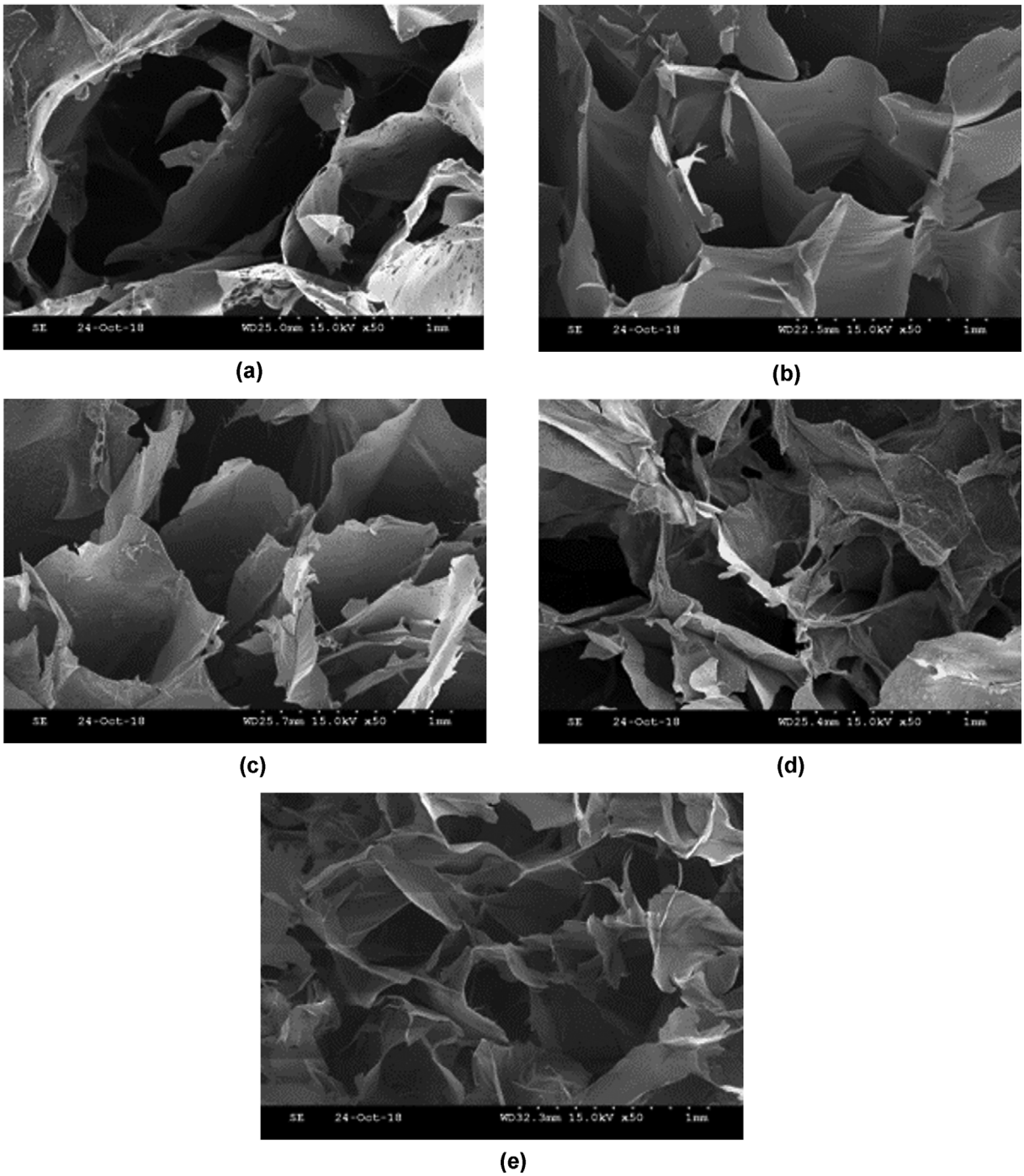

Fig. 3. SEM image of HPMC/gellan gum hydrogels at 50× magnification. (a) H5/G5, (b) H4/G6, (c) H3/G7, (d) H2/G8, and (e) H1/G9.

To examine the whitening effect of quercetin, the melanin content of B16F10 melanoma cells, induced with α-MSH (1 μM) for melanin production, was measured after treatment with quercetin (10-100 μM) (Fig. 6(b)). At the total concentration tested, melanin levels were reduced compared to those in the control group, especially when the quercetin concentration was 20 μM. Interestingly, similar quercetin at 20 μM and higher concentrations of 50 μM, 80 μM, and 100 μM showed similar reductions in melanin, with quercetin at 20 μM, 50 μM, 80 μM, and 100 μM showing 59.45%, 56.54%, 55.89%, and 55.63% of the melanin content, respectively. The IC_{50} value of quercetin obtained from the experiment was 98.18 μM; however, the IC value is not reliable because the quercetin inhibitory effect on melanin formation is independent of concentration.

4. Water Uptake of Hydrogels

The water absorption capacity of the hydrogel, concentration of HPMC in swelling, and concentration ratio of gellan gum used as a cross-linker are important conditions. In this study, the water

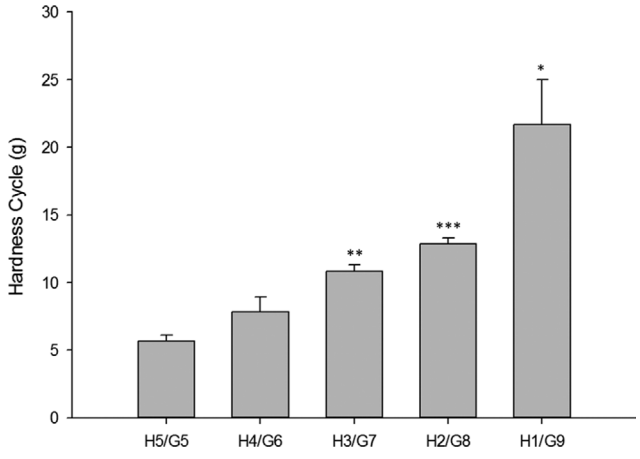

Fig. 4. Effect of HPMC and gellan gum concentration ratio on strength. The total solid concentration of all hydrogels was 4% (w/v), and $CaCl_2$ was added at the same concentration of 6 mM. The results were compared using the H5/G5 hydrogel with the lowest gellan gum concentration ratio as the control (n=4, mean±SD, *: p<0.05, **: p<0.01 and ***: p<0.005).

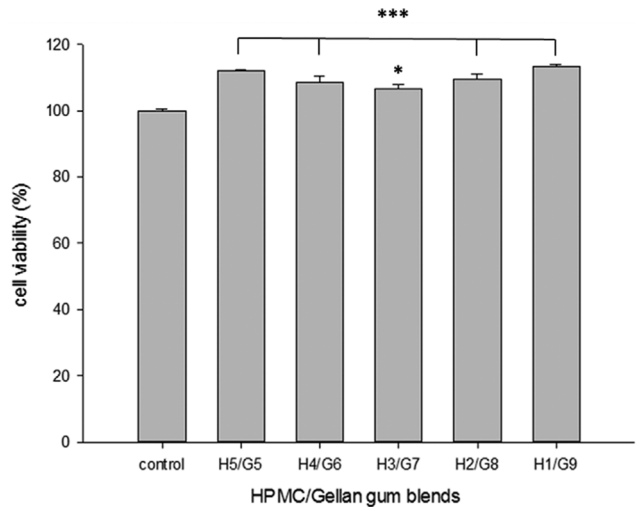

Fig. 5. Effect of HPMC/gellan gum hydrogel extract on B16F10 cells. Cells were exposed to each concentration of HPMC/gellan gum hydrogel extract for 24 h. Hydrogel extract-treated cells were compared with untreated controls (n=3, mean±SD, *: p<0.05, **: p<0.01, and ***: p<0.005).

absorption capacity of HPMC/gellan gum hydrogels prepared at different concentration ratios was used to control quercetin release (Fig. 7) and was as follows: G1 hydrogel (3.86%)>H2/G8 hydrogel (3.86%)>H1/G9 hydrogel (8.74%)>H3/G7 hydrogel (5.14%). The water absorption capacity of the H5/G5 hydrogel (with the lowest ratio of gellan gum) was approximately 3.67 times higher than that of H1/G9 (with the highest ratio of gellan gum).

5. *In vitro* Quercetin Release

The drug release studies of quercetin in the HPMC/gellan gum hydrogel are shown in Fig. 8. Under all conditions, the hydrogel rapidly increased the amount of quercetin released for 2 h after the start of the release experiment. However, from 2 h onwards, the HPMC/

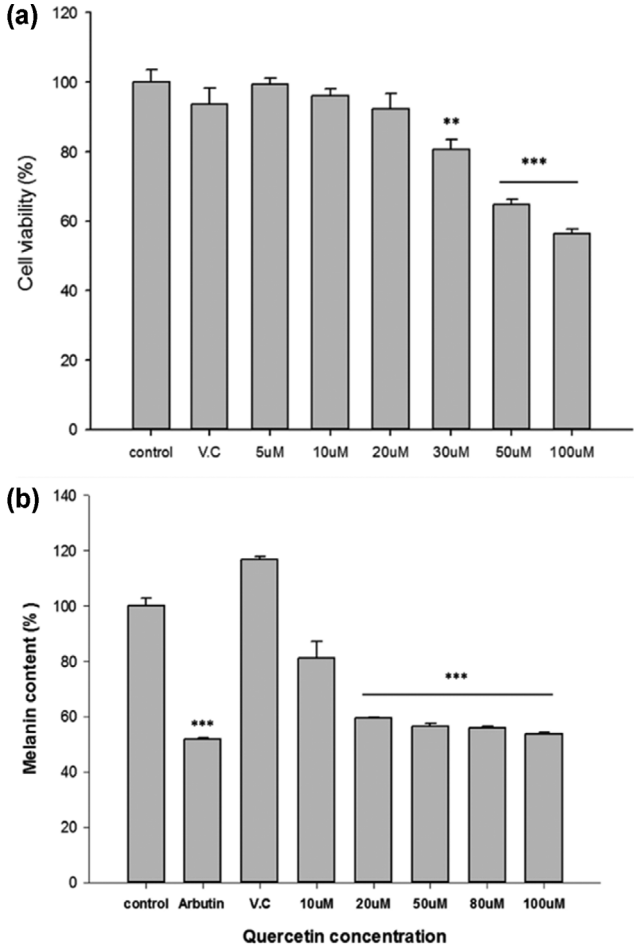

Fig. 6. Effects of quercetin on B16F10 cells. B16F10 cells were treated with quercetin at a concentration of 5-100 μM for 24 h. The results were compared with untreated control cells. The vehicle control (V.C.) was treated with 0.025% (w/v) DMSO as a quercetin solvent. (a) Cell viability with respect to quercetin. (b) Melanin content with quercetin concentration. All cells used in the melanin assay induced α-MSH to induce melanogenesis, and arbutin was used as the positive control (P.C.) (n=3, mean±SD, *: p<0.05, **: p<0.01, and ***: p<0.005).

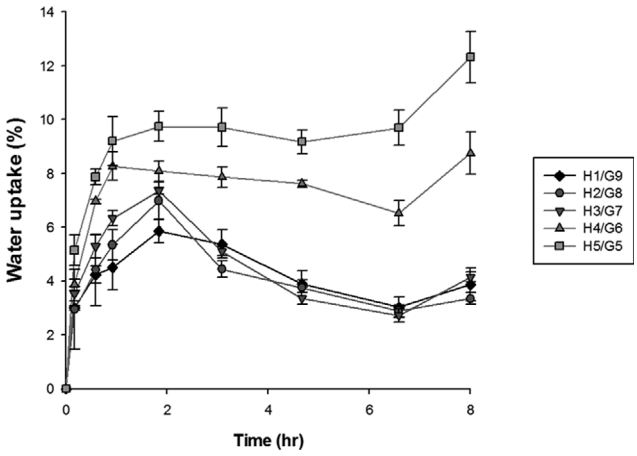

Fig. 7. Water uptake by the HPMC/gellan gum hydrogel (n=3, mean±SD).

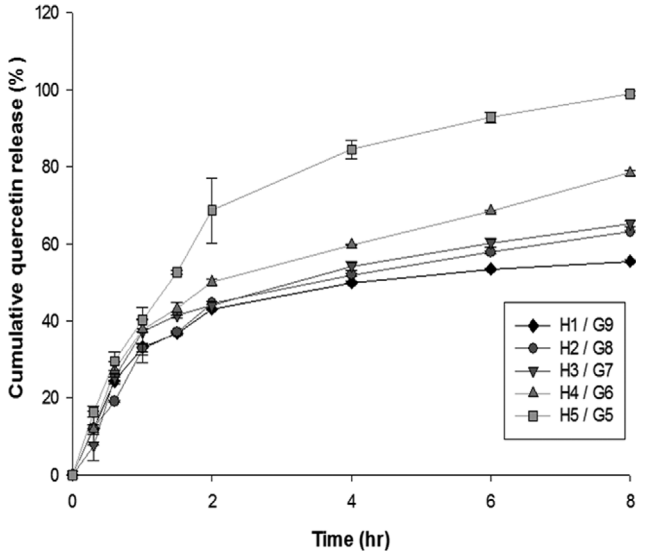

Fig. 8. Cumulative release of quercetin from HPMC/gellan gum hydrogels (n=3, mean±SD).

gellan gum composition changed and quercetin release also changed in the hydrogel. The hydrogel with the highest amount of released quercetin was H5/G5. H5/G5 continued to show high amounts of quercetin release after 2 h, and 99.08±0.649% of quercetin was released after 8 h. Further, the H4/G6 hydrogel showed quercetin release of 78.47±0.625% after 8 h. Quercetin release from the H3/G7, H2/G8, and H1/G9 hydrogels after 8 h was 65.24±0.817%, 63.19±0.192% and 55.48±0.288%, respectively.

DISCUSSION

Addition of calcium chloride to the HPMC/gellan gum achieves a dual effect. First, anions, such as chloride, have a strong salt-out effect [12]. The salt-out effect increases the hydrophobic interaction of the HPMC solution, allowing gelation at low temperatures and shortening the gelation time. Second, Ca^{2+} cross-links the cation-binding sites in gellan gum [14]. Crosslinking of gellan gum and Ca^{2+} reduces the pore size of the gel and increases stiffness by forming a dense gel structure. In the experimental results, the gelation time decreased as the ratio of gellan gum increased, presumably because of the salt-out effect of calcium chloride, as described above, or because of the salt-out effect caused by the anionic polysaccharide, gellan gum. Another reason is that as the ratio of gellan gum increases, the gelation time is shortened because it can cross link with more Ca^{2+}. SEM measurements indicated that HPMC/gellan gum hydrogels under all conditions were porous, and that the pore size of the hydrogel decreased as the concentration ratio of added gellan gum increased. The SEM image revealed an increasingly dense network of small, more detailed pores with the increasing addition of gellan gum. Gellan gum can undergo gelation by cross-linking with Ca^{2+}, and increased crosslinking reduces the hydrogel pore size [15]. Stiffness was significantly increased depending on the gellan gum concentration, which can be related to the pore size as described above. This is because a larger pore size will have a hydrogel structure with a looser network, whereas a smaller, uniform pore size will increase the hydrogel density and rigidity.

Quercetin cytotoxicity was not significantly different from that reported previously. Our results indicate that cell viability decreased in a concentration-dependent manner at quercetin concentrations higher than 20 μM. These results are similar to those of previous studies in which B16F10 melanoma cells treated with quercetin did not show cytotoxicity at concentrations below 20 μM [16]. To date, the cell viability of quercetin has been reported to be below 20 μM in most studies [8]. These findings suggest that quercetin is highly cytotoxic and induces apoptosis [17].

Quercetin inhibits tyrosinase, the most important component of the melanin synthesis pathway, by acting as a copper chelator [5]. In the melanin assay, quercetin decreased melanin production at 20 μM and showed similar inhibition of melanin production as that of arbutin, which was used as a positive control. Fujii et al. reported similar results, suggesting that inhibition of the synthesis of tyrosinase, a typical melanogenesis inducer, in melanoma cells treated with quercetin 20 μM resulted in decreased melanin production [18]. The cell viability and anti-melanogenesis results showed that when the quercetin concentration was 20 μM, there was no cytotoxicity or melanin content reduction. Therefore, we set the optimum concentration of quercetin to 20 M for practical application. To gradually release quercetin at the optimal concentration, we designed a method to apply the HPMC/gellan gum hydrogel as a carrier. We performed cell viability, water uptake, and release experiments using HPMC/gellan gum hydrogels to control quercetin release and evaluated their potential as carriers.

Toxicity assessment is essential for applying HPMC/gellan gum hydrogels to the skin. Therefore, *in vitro* cell viability tests were performed using the well-known WST-1 cytotoxicity assay. When the total solid concentration of the hydrogel was 8% (w/v) with 6 mM $CaCl_2$, the cells exhibited adequate stiffness but high toxicity. Thus, the concentration of the total solids in the hydrogel was reduced to 2-4% (w/v), and the addition of 6 mM $CaCl_2$ doubled the stiffness. As shown in Fig. 5, the final total solid content of the hydrogel was 4% (w/v), and the $CaCl_2$ concentration was 6 mM. This result indicates that HPMC/gellan gum hydrogel can be safely applied to the human body, indicating that it is an appropriate carrier.

Hydrogel swelling and water absorption capacity are important factors in drug release using hydrogels [10]. Fig. 8 shows that the concentration of HPMC and gellan gum, used as a cross-linker, showed different water absorption capacities. The water absorption capacity of the H1/G9 hydrogel (with the highest ratio of gellan gum) was lower than that of H5/G5 (with the lowest ratio of gellan gum). This difference was approximately 3.67 times. The water absorption in the hydrogel varies significantly with the hydrodynamic free volume [19]. An increase in the crosslinker, gellan gum, induced cross-linking with calcium ions and reduced the pore size of the gel. Therefore, as the concentration ratio of gellan gum increases, the porosity of the hydrogel decreases, resulting in a lower hydrodynamic free volume. This was presumably the reason for the lower water absorption capacity of the H2/G8 and H1/G9 hydrogels with relatively high concentrations of gellan gum. Conversely, when the concentration ratio of gellan gum was low (i.e., the crosslink density was low), the network loosened and had a high hydrody-

namic free volume, which could accommodate more solvent molecules, causing the matrix to expand. For this reason, H4/G6 and H5/G5 hydrogels, which have relatively low gellan gum concentration ratios, can absorb a large amount of water.

Quercetin has a strong inhibitory effect on melanin production; however, it is too toxic to be used as a whitening drug. Therefore, further research is needed for continuous release of quercetin at a concentration that has a whitening effect without toxicity. In the drug delivery system, when HPMC hydrogel is used as the carrier, it expands at 37 °C and releases the drug [20]. We devised a way to control the rate of drug release in HPMC by adding a cross-linker, gellan gum. In our drug release studies, the release rate of quercetin decreased when the concentration of gellan gum was high. The quercetin release of the H1/G9 hydrogel was approximately 1.78 times less than that of the H5/G5 hydrogel over 8 h. As drug release through the hydrogel is determined by gel expansion, the SEM and water uptake results described above can explain the drug release results. The SEM experiment showed that the hydrogel with a high concentration of gellan gum had a dense hydrogel structure composed of small and uniform pores, and the decreased porosity resulted in inferior expansion of the hydrogel, with decreased water uptake rates. These results suggest that drug release of quercetin is greatly influenced by a decrease in pore size owing to an increase in the concentration of the cross-linker gellan gum. After quercetin loading on gelatin-carrageenan hydrogel, the initial release of quercetin is accelerated as the pore size increases [21]. In conclusion, the addition of HPMC with high gellan gum concentration can reduce the pore size and swelling of the hydrogel through cross-linking and reduce the release rate of quercetin.

CONCLUSIONS

We designed an HPMC/gellan gum hydrogel that can control the continuous release of the whitening agent quercetin at a non-toxic concentration, and investigated the biophysical properties of the hydrogel. The concentration of quercetin used was 20 μM, which showed a good whitening effect and no toxicity. Cell viability experiments using the five HPMC/gellan hydrogel extracts demonstrated that none of the hydrogels were toxic, indicating the safety of the HPMC/gellan hydrogel. The hydrogel property that controlled the release of quercetin most effectively was the HPMC/gellan gum ratio. The cumulative release of quercetin (after 8 h) followed the order: H5/G5 hydrogel (99.08%)>H4/G6 hydrogel (78.47%)>H3/G7 hydrogel (65.24%)>H2/G8 hydrogel (55.48%). Overall, HPMC/gellan gum hydrogels were non-toxic *in vitro* and effectively controlled the release of quercetin. In particular, when the concentration of gellan gum as a cross-linker was high, quercetin release was controlled effectively, owing to the decrease in pore size, water uptake, and swelling by the increased crosslinking. As the results of this study were obtained from *in vitro* assays, further *in vivo* studies are needed.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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